

**AWARD NUMBER:** W81XWH-15-1-0640

**TITLE:** Novel Autoantibody Serum and Cerebrospinal Fluid Biomarkers in Veterans with Gulf War Illness

**PRINCIPAL INVESTIGATOR:** Kimberly Sullivan, Ph.D.

**RECIPIENT:** Trustees of Boston University  
Boston, MA 02118

**REPORT DATE:** October 2016

**TYPE OF REPORT:** Annual

**PREPARED FOR:** U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

**DISTRIBUTION STATEMENT:** Approved for public release; distribution unlimited

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| REPORT DOCUMENTATION PAGE   |              |                          |                            | Form Approved<br>OMB No. 0704-0188            |   |
|---|--------------|--------------------------|----------------------------|---|---|
| Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.  |              |                          |                            |   |   |
| 1. REPORT DATE<br>October 2016  |              | 2. REPORT TYPE<br>Annual |                            | 3. DATES COVERED<br>30 Sep 2015 - 29 Sep 2016 |   |
| 4. TITLE AND SUBTITLE<br><br>Novel Autoantibody Serum and Cerebrospinal Fluid Biomarkers in Veterans with Gulf War Illness  |              |                          |                            | 5a. CONTRACT NUMBER                           |   |
|   |              |                          |                            | 5b. GRANT NUMBER<br>W81XWH-15-1-0640          |   |
|   |              |                          |                            | 5c. PROGRAM ELEMENT NUMBER                    |   |
| 6. AUTHOR(S)<br>Kimberly Sullivan, Ph.D.<br><br>E-Mail: tty@bu.edu  |              |                          |                            | 5d. PROJECT NUMBER                            |   |
|   |              |                          |                            | 5e. TASK NUMBER                               |   |
|   |              |                          |                            | 5f. WORK UNIT NUMBER                          |   |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br>Trustees of Boston University<br>Boston, MA 02118   |              |                          |                            | 8. PERFORMING ORGANIZATION REPORT NUMBER      |   |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br><br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012   |              |                          |                            | 10. SPONSOR/MONITOR'S ACRONYM(S)              |   |
|   |              |                          |                            | 11. SPONSOR/MONITOR'S REPORT NUMBER(S)        |   |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br><br>Approved for Public Release; Distribution Unlimited  |              |                          |                            |   |   |
| 13. SUPPLEMENTARY NOTES<br>None   |              |                          |                            |   |   |
| 14. ABSTRACT<br>The purpose of this project is to develop objective peripheral biomarkers for Gulf War Illness (GWI). We plan to determine circulating autoantibodies to ten proteins associated with the central nervous system in sera and plasma from a group of 250 Gulf War veterans with Gulf War Illness (GWI) and from 200 controls (100 healthy GW controls, 100 disease controls). Preliminary results using Western blot assay showed increased levels of autoantibodies in GWI cases compared to symptomatic controls in the following descending order: CaMKII > GFAP > Tau > Tubulin > MAG > MAP-2 > MBP > NFP > S100B ranging from 2-8 fold higher. These results confirm the continuing presence of autoantibodies against neuronal and glial cells in these veterans and are in agreement with recent reports indicating that 25 years after the war, the health of veterans with GWI is not improving and may be getting worse. Such blood-based autoantibodies may prove useful as biomarkers of GWI, upon validation of the findings using our collective larger cohorts. |              |                          |                            |   |   |
| 15. SUBJECT TERMS autoantibodies, Gulf War Illness, central nervous system, biomarkers, irritable bowel syndrome, chronic fatigue syndrome  |              |                          |                            |   |   |
| 16. SECURITY CLASSIFICATION OF:   |              |                          | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES                           | 19a. NAME OF RESPONSIBLE PERSON           |
| a. REPORT   | b. ABSTRACT  | c. THIS PAGE             |                            |   | USAMRMC                                   |
| Unclassified  | Unclassified | Unclassified             | Unclassified               | 12  | 19b. TELEPHONE NUMBER (include area code) |

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## 1. Introduction

**Subject:** The focus of this study is the assaying of autoantibodies against neuronal and glial proteins in blood samples from veterans with Gulf War Illness (GWI) compared with healthy and symptomatic control groups (CFS/IBS).

**Purpose:** The purpose of this study is the development of objective peripheral blood biomarkers of GWI that are indicative of autoantibodies from the CNS as a result of at least a 1-time leaky blood brain barrier (BBB).

**Scope of the Research:** Serum and plasma from 250 Gulf War veterans with GWI and 200 controls (100 healthy GW veterans; 50 chronic fatigue syndrome (CFS) and 50 irritable bowel syndrome (IBS) non-veterans) will be analyzed in this study from several current and prior studies using stored blood samples.

## 2. Key Words

Gulf War Illness, MAP-2, tubulin, NFP, tau, MBP, MAG, CaMKII, GFAP, S100B, Western Blot, ELISA, chronic fatigue syndrome, irritable bowel syndrome

## 3. Accomplishments

- What were the major goals of the project?
  - The major goals of the project as stated in the approved SOW for year 1 are listed in the table below. Specifically, during year 1, the primary goals were to obtain local and DOD approvals for use of the stored blood samples and coordinating the shipping of the samples to the lab at Duke University for CNS autoantibody analysis. Milestones/target dates for important activities or phases of these dates and actual completion dates are listed in the table below.
- What was accomplished under these goals?
  - The table lists the proposed goals and dates for accomplishment and the actual dates that these goals were accomplished during the first 12-month period. The text below provides additional details of accomplishments.

| Tasks  | Timeline       |               |
|--|----------------|---------------|
| Task 1: Obtain Regulatory Reviews and Approvals  | Planned Months | Actual Months |
| 1a. Obtain necessary IRB approvals or Exempt status  | 1-3            | 1-4           |
| 1b. Obtain DOD Human Research Protections Office (HRPO) approvals or Exempt Status                           | 1-3            | 1-4           |
| Milestone(s) Achieved: Regulatory reviews completed and final approval obtained for study                    | 1-3            | 1-4           |
| Task 2: Obtain Stored Blood Serum and CSF samples from 3 biorepositories for analysis.                       | Months         |               |
| <b>Proposed 2a:</b> 100 GWI and 50 IBS serum samples shipped from Site 5 to Duke and NIH for analysis.       | 4-6            |               |
| <b>Actual 2 a:</b> 100 GWI and 50 IBS <i>plasma</i> samples shipped from Site 5 to Duke and NIH for analysis |                | 6-8           |
| <b>Actual 2a:</b> Adapting western blot for plasma samples   |                | 10-11         |
| <b>Actual 2a:</b> Beginning analysis of all plasma samples from 100 GWI and 50 controls.                     |                | 10-12         |

|  |        |  |
|--|--------|--|
| <b>Proposed 2b:</b> 50 GWI and 50 healthy GW veteran controls and 50 CFS control <b>serum</b> samples shipped from site 4 to Duke and NIH for analysis.<br><b>Actual 2b:</b> <i>Plasma samples received from NOVA University:</i><br>1. 50 CFS samples<br>2. 26 healthy GW control samples<br>3. 68 GWI samples (from Boston GWIC) | 4-9    | 12   |
|  |        |  |
| *2c: 100 GWI and 50 healthy GW veteran control <b>serum</b> samples shipped from site 1 to Duke and NIH for analysis.  | 10-22  |  |
| <b>Proposed</b> *2d: 25 GWI and 25 healthy GW veteran control CSF samples shipped from site 1 to NIH for analysis  | 22-24  |  |
| Milestone(s) Achieved: Site 1, 4 and 5 serum and CSF data collected and set up for laboratory assays (ELISA, western blot). Autoantibody data shipped to analyzing labs from 250 GWI veterans and 200 controls (100 healthy and 100 diseased controls) blood serum samples and 50 CSF (25 GWI, 25 control) samples.                | 3-24   | Samples were shipped to Duke lab for analysis from three sites including 294 samples (168 GWV; 50 IBS; 50 CFS) |
| <b>Task 3: Perform Serum Assays</b>  | Months |  |
| 3a: Perform western blot analyses for autoantibodies to CNS proteins in GWI cases and control samples.   | 4-24   |  |
| 3b: Perform ELISA analyses for Neurofascin 155 CNS marker in serum samples from GWI cases and controls.  | 3-24   |  |
| Milestone(s) Achieved: Autoantibodies for CNS proteins of myelinogenesis, astroglioneogenesis and neurogenesis data analyzed from three biorepository sites.   | 9-24   |  |
| <b>Task 4: Perform CSF Assays</b>  | Months |  |
| 4a: Perform ELISA assays of 50 CSF samples for neurofascin 155 biomarker.  | 22-24  |  |
| 4b: Merge CSF outcome data with clinical neuroimaging, TBI and exposure data.  | 24-27  |  |
| Milestone(s) Achieved: Antibody for neurofascin 155 marker data analyzed and merged with clinical outcome data from GWIC biorepository site.   | 24-27  |  |
| <b>Task 5: Merge Data and Perform Interim Data analyses</b>  | Months |  |

|  |        |  |
|--|--------|--|
| 5a: Merge clinical dataset data from sites 1, 4, 5 case/control status and demographics with results from laboratory analyses performed at NIH and Duke.   | 10-24  |  |
| 5b: Data analysis of interim ELISA and western blot results of autoantibodies in GWI cases and controls (healthy and diseased groups) with merged clinical datasets.   | 18-24  |  |
| 5c: Discussion of results and preparation of abstracts for meeting presentations and initial manuscript for publication.   | 18-24  |  |
| 5d. Annual reports of progress will be written.  | 12-24  |  |
| Milestone(s) Achieved:<br>Preliminary analysis of results and presentation of initial results at scientific meetings and potential publication.<br>Possible biomarker selection for GWI and recommendations for treatment development.   | 18-24  |  |
| <b>Task 6: Perform Final Data analyses and Prepare Manuscripts for Publication</b>   | Months |  |
| 6a: Merge clinical datasets for neuroimaging, blood and genetic biomarkers, brain injury and exposure history with GWI cases and controls.   | 25-30  |  |
| 6b: Perform Data analysis comparing ELISA and western blot autoantibodies outcomes in GWI cases and controls with merged clinical datasets for neuroimaging, blood and genetic biomarkers, brain injury and exposure history.  | 25-30  |  |
| 6c: Discuss results of data analyses and prepare abstracts for DOD and other scientific meetings.  | 25-32  |  |
| 6d: Preparation of manuscripts <ul style="list-style-type: none"> <li>• Diagnostic CNS Autoantibody Biomarkers of GWI</li> <li>• Biomarkers of OP pesticide and nerve agent exposures in GW veterans</li> <li>• Biomarkers of prior brain injury in GW veterans</li> </ul>   | 25-36  |  |
| 6e: Final report of progress will be written.  | 35-36  |  |
| Milestone(s) Achieved:<br>Analysis of all study results, presentation of results at scientific meetings, submitted publication and final report of progress.<br>Possible diagnostic biomarker selection for GWI, brain injury and deployment-related exposures and potential recommendation for treatment development. | 35-36  |  |

\* Serum and CSF samples will be collected as part of the ongoing Boston GWI consortium study and will be sent to NIH and Duke study sites as the samples are added to the GWIC biorepository. These samples will all be collected by month 24 of the current study.

- **What was accomplished under these goals?**
- **Task 1: Obtain Regulatory Reviews and Approvals**
  - Local IRB approval at Boston University School of Public Health and Duke University were obtained and then DOD HRPO approval was also obtained for both sites in the first several month start-up period of the study.
- **Task 2: Obtain Stored Blood Serum and CSF samples from 3 biorepositories for analysis.**
  - **Major activities: Coordinated sending blood samples from nearly 300 Gulf War veterans and symptomatic controls from three study sites to Duke Laboratory for analysis.** Blood samples were shipped to the Duke lab for analysis from three study sites including 294 samples (168 GWV; 50 IBS; 50 CFS). BUSPH investigators are developing a REDCap web-based data collection system for the blood based results and corresponding demographic information from the three different study sites. It was also determined during early discussions with other co-investigators that more plasma than serum was available at the study sites so a large aim of this period was to adapt the Western blot assay of autoantibodies from serum samples to plasma samples so that both could be used in this study and importantly in other validation studies and clinical assessments in the future.
  - **Specific objectives:** Duke investigators established Western blot analysis for autoantibodies against neural proteins in plasma samples from GWI subjects and from controls similar to our original method in serum samples from a small pilot sample that showed similar results.
  - **Significant results:** These results now allow the running of autoantibody assays not only in serum but also in plasma. This is a considerable advantage because many specimens from GW veterans are prepared as plasma not serum and other stored sample studies could now be used for this purpose.
  - **Other achievements.** Currently, BUSPH investigators are working on finalizing the REDCap database for this study so that data can be shared between the groups and easily sent to the study statistician for planned analyses. Duke investigators are continuing to adapt our autoantibody assays against neural proteins to ELISA assays. This will give another dimension in assaying large number of samples to these biomarkers.
- What opportunities for training and professional development has the project provided?
 

*Professional development.* The results of studies that were generated in this project were presented in a national meeting and an international meeting reported below. In addition, a BUSPH doctoral student is participating in a research rotation to assist with developing the REDCap database and preliminary analyses for the study.

  1. The Annual Meeting of the Society of Toxicology, March 2016, New Orleans: A poster of the results was presented.
  2. The International Neuropsychological Society (INS) annual mid-summer meeting, July 2016, London UK; an oral presentation was given as part of an invited symposium on Gulf War Illness organized by Dr. Sullivan.
- How were the results disseminated to communities of interest?
  - Our results were discussed with many interested scientists during both the SOT and INS meetings.
  - A symposium on the Gulf war Illness chaired by Dr. Sullivan took place in London during the International Neuropsychological Society mid-year meeting last July, during which results from this project and several other related Boston GWI consortium projects were presented and stirred intense interests.

- What do you plan to do during the next reporting period to accomplish the goals?
  - We plan to attend appropriate National and International meetings to present our results from this project.
  - Annual Meeting of the Society of Toxicology, March 2017, Baltimore, Maryland. An abstract has been submitted for a poster presentation.
  - We submitted a manuscript of the pilot study to the Journal Neurotoxicology and Teratology and are awaiting the reviewer comments. We hope that this manuscript will be accepted during the next reporting period.
  - We plan to submit other manuscripts of preliminary results from the case-control and exposed vs non-exposed groups during the next reporting period as well.

#### 4. Impact

- What was the impact on the development of the principal discipline(s) of the project?
  - Our studies have focused on the development of sensitive, specific, reproducible, and non-invasive blood biomarkers of GWI. Identifying objective biomarkers of GWI will help the veteran and the treating clinicians who must now rely on self-report of symptoms as the primary diagnostic marker. The advantage of a blood-based biomarker is that it can diagnose GWI with greater accuracy and with only a few drops of blood. Our initiative to validate both serum and plasma for these potentially diagnostic autoantibodies will make diagnosing GWI and validating it with other stored blood samples from GWI even easier because it won't be limited by just one type of blood product.
  - The results of the study can be applied immediately to treatment development strategies for the veterans of the Gulf War. Based on the CNS autoantibodies we ultimately find, this will provide the opportunity to develop drugs that treat neuronal injury or neuroinflammation in those specific pathways (neuronal, glial etc); such treatment could be directly applicable to Gulf War veterans in the short-term.
- What was the impact on other disciplines?
  - A major advantage of our peripheral marker is that it is specific for neural injury irrespective of the cause, thus it can be applied to diagnose or confirm diagnosis of other neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease or perhaps also chronic fatigue syndrome. However, the *pattern* of the specific markers elevated can be different with different disease states and thus can also be useful diagnostically once validated in different disorders.
  - Blood-based biomarkers of GWI provide an effective way to enhance its management
    - It can be used as a diagnostic and prognostic tool with the ability to provide information about rate of disease progression.
    - It would help in identification of novel and effective treatments for multiple disorders and environmental exposure groups (i.e. pesticides, nerve agents).
    - It could be used for monitoring therapeutic efficacy for the benefit of patients and caretakers.
    - It could be used to follow-up treatment plans of the patient
    - It could provide a cost-savings potential for recruitment into clinical trials.
- What was the impact on technology transfer?
  - The biomarker that we developed is cost effective, available, and does not need expensive technicians that can be used to diagnose neural injury without the need for expensive equipment.



- What was the impact on society beyond science and technology?
  - The hallmark of Gulf War Illness (GWI) is neuroinflammation, neural cell death in specific regions of the brain and possible progressive neurodegeneration. A challenging aspect of GWI is that it has been difficult to diagnose with objective biomarkers because organophosphate pesticides and nerve agents do not stay in the body and CNS the same way that other exposures do (i.e. agent orange, depleted uranium, lead, mercury). Therefore, researchers have had to develop markers of damage from these chronic exposures rather than markers of the exposure or their bi-products. If successful, this work will impact neurotoxicant exposed individuals including agricultural workers, pesticide applicators and nerve gas exposed groups by providing objective inexpensive markers of chronic damage related these exposures that can be conducted virtually anywhere that a simple blood draw can be obtained and analyzed. Other current diagnostic practices including neuroimaging techniques, behavioral history assessments, and neuropsychiatric tests have drawbacks of not always being practical or available in other parts of the world but a simple blood test could provide objective diagnostic markers in the most cost-effective way.

## **Changes/Problems**

- Changes for approach and reasons for change
  - Changes: serum to plasma samples  
Our original studies in determining autoantibodies in blood used serum samples from GWI cases and symptomatic controls. However, it was determined that our co-investigators had more plasma available than serum. Therefore, we carried out experiments to establish the validity of our assay using plasma, as stated above under Accomplishments, which showed that the results from plasma samples were identical to those of serum samples. This was a big accomplishment that either plasma or serum can be used for these analyses thus all other major studies of GWI with either serum or plasma samples could potentially validate our findings with their own samples.
  - Problems: none
- Actual or anticipated problems or delays and actions or plans to resolve them techniques
  - None is actual or anticipated
- Changes that had significant impact on expenditures
  - None
- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:
  - Significant changes in use or care of human subjects: None
  - Significant changes in use or care of vertebrate animals: None
  - Significant changes in use of biohazards, and/or select agents: None

## 5. Products

- **Publications, conference papers, and presentations**

- **Journal Publications**

A manuscript was prepared and submitted for publication to the journal Neurotoxicology and Teratology. A revised version was recently submitted and we are waiting for the editorial decision. The

manuscript title is: “Screening for Novel Central Nervous System Biomarkers in Veterans with Gulf War Illness”

- **Books or other non-periodicals, one-time publications**
  - None
- **Other publications, conference papers, and presentations**
  - A poster was presented during the annual Society of Toxicology Meeting at New Orleans entitled: “A Pilot Study of Novel Brain Neurodegenerative Biomarkers in Veterans with Gulf War Illness”. MB. Abou-Donia, K Sullivan, L A. Conboy, and E Kokkotou.
  - An oral presentation was given in a Symposium on Gulf War Illness during the International Neuropsychological Society (INS) annual mid-year meeting in July, 2016 entitled: “A Pilot Study of Novel Brain Neurodegenerative Biomarkers in Veterans with Gulf War Illness: M. B. Abou-Donia, K. Sullivan, L. A. Conboy, E. Kokkotou and E. M, El-Masry.”

**Other products**

- **Website(s) or other Internet site(s)**
  - None
- **Technologies or techniques**
  - None
- **Other products**
  - None

**6. Participants & other Participating Organizations**

Site 1: Boston University School of Public Health  
715 Albany Street, T4W  
Boston, MA 02118  
Initiating PI: Dr. Kimberly Sullivan  
Co-I: Dr. Joseph Massaro  
Co-I: Dr. Maxine Krengel  
Tasks 1-6

Site 2: Duke University Medical Center  
Durham, North Carolina 27710  
Partnering PI: Dr. Mohamed Abou Donia  
Co-I: Dr. Cameron R. ‘Dale’ Bass  
Tasks 2,3,5,6

Site 3: National Institutes of Health, NICHD  
Bldg. 35, Room 2A211, MSC 3713  
35 Lincoln Drive  
Bethesda, MD 20892

Site PI: Dr. R. Douglas Fields  
Co-I: Dr. Dipankar Dutta  
Tasks 2-6

Blood Serum and CSF Biorepository Sites

Site 4: NOVA Southeastern University  
Ft. Lauderdale, FL  
Co-I: Dr. Nancy Klimas  
Tasks 1,2, 5, 6

Site 5: Beth Israel Deaconess Medical Ctr.  
Boston, MA 02118  
Consultant: Dr. Efi Kokkotou  
Consultant: Dr. Lisa Conboy  
Tasks 1,2, 5,6

## **Study Sites Responsibilities**

**Site 1:** Dr. Sullivan and her BUSPH team will be responsible for providing the serum blood and cerebrospinal fluid samples from GWIC study participants who have agreed to share their specimens with the GWIC biorepository to be used in future studies including the proposed study. Specifically, she will oversee the recruitment and blood draws/lumbar punctures of study participants from the GWIC study and the processing of serum and CSF samples that will be shared for the proposed study. Dr. Sullivan will also assist with the experimental design, data analysis and interpretation and presentation of study results in collaboration with Dr. Abou Donia and the other study investigators. **Tasks 1-6**

**Site 2:** Dr. Abou-Donia will be responsible for receiving the serum samples from all sites and performing autoantibody analyses using western blot analyses for 450 serum samples (250 GWI, 200 controls). He will also assist with the experimental design, interpretation of data, report and manuscript writing and presentation of results at scientific meetings. **Tasks 2, 3, 5, 6**

**Site 3:** Dr. Fields will be responsible for receiving the serum and CSF samples from all study sites and performing ELISA assays for 450 serum samples and 50 CSF samples. He will assist with the experimental design, interpretation of data, report and manuscript writing and presentation of results at scientific meetings. **Tasks 2-6**

**Sites 4 and 5:** Drs. Klimas, Conboy and Kokkoutu will provide serum samples from their respective biorepositories for study analyses, will assist with interpretation of data, report and manuscript writing.

### **7. Special Reporting Requirements**

None

### **8. Appendices**

Appendix 1:

Abstract 1. An Abstract that was presented as a poster during the society of toxicology annual meeting in March 2016, in New Orleans Louisiana.

Abstract 2. An Abstract that was presented during the International Neuropsychological Society meeting in London, UK

## Appendix 1

### Abstract 1.

#### A. Annual Society of Toxicology Meeting, New Orleans, LA, March, 2016

A Pilot Study of Novel Brain Neurodegenerative Biomarkers in Veterans with Gulf War Illness. MB. Abou-Donia<sup>1</sup>, K Sullivan<sup>2</sup>, L A. Conboy<sup>3</sup>, and E Kokkotou<sup>4</sup>

<sup>1</sup>Duke University Medical Center, Durham, NC, <sup>2</sup> University of Boston, Boston, MA, <sup>3</sup>Harvard Medical School, Boston, MA, <sup>4</sup>Harvard Medical School. Boston, MA

Upon their return from the 1990-1991 Gulf War (GW), hundreds of thousands of American military personnel complained of symptoms with unknown etiology, known as the Gulf War Illness (GWI). The hallmark of GWI is neural degeneration that is consistent with symptoms related to nervous system injury and confirmed by experimental studies. A major problem in identifying veterans with GWI is the difficulty in its diagnosis. This report presents the results of a pilot, investigative and descriptive study of assays performed to detect circulating autoantibodies to a panel of nine proteins associated with the nervous system in sera of a group of 20 veterans of the Gulf War who reported having symptoms of Gulf War Illness (GWI) and 10 symptomatic controls who did not have GWI. Various types of proteins present in axons, dendrites and myelin sheath that are affected by neuronal degeneration were used. In sera samples from the GWI subjects and symptomatic non-veteran controls using Western blotting, immunoglobulin IgGs were measured against: neurofilament triplet proteins (NFP), tubulin, microtubule associated tau proteins (tau), microtubule associated protein-2 (MAP-2), myelin basic protein (MBP), myelin associated glycoprotein (MAG), glial fibrillary acidic protein (GFAP), calcium-calmodulin kinase -2 (CAM-2) and glial S100B protein. Also,  $\alpha$ -synuclein, a marker for Parkinson' disease was included. The results show significantly elevated levels of circulating Ig-G-class autoantibodies in the veterans with GWI, compared to controls. This preliminary study demonstrates a relationship between clinical condition, and the level of serum autoantibodies to nervous system-specific proteins. These results showing the development of neuronal injury and gliosis in the subjects are consistent with recent reports indicating 20 years after the Gulf War, the health of the- veterans who developed GWI is getting worse. It is concluded that that these serum circulating autoantibodies may be used as biomarkers for confirming GWI upon further validation. (Supported in part by DOD Contracts No. W81XWH-15-1-0641 and W81XWH-15-1-0640).

### Abstract 2.

#### B. International Neuropsychological Society (INS) annual mid-year meeting, July 2016.

London Abstract: A Pilot Study of Novel Brain Neurodegenerative Biomarkers in Veterans with Gulf War Illness: M. B. Abou-Donia<sup>1</sup>, K. Sullivan<sup>2</sup>, L. A. Conboy<sup>3</sup>, E. Kokkotou<sup>4</sup>, and E. M. El-Masry<sup>5</sup> Duke University Medical Center, Durham, NC, <sup>2</sup> Boston University School of Public Health, Boston, MA, <sup>3</sup>Harvard Medical School, Boston, MA, <sup>4</sup> Harvard Medical School. Boston, MA ; <sup>5</sup> Zagazig University, Zagazig, Egypt

Objective: To determine circulating autoantibodies in ten proteins associated with nervous system in sera of a group of 20 veterans of the Gulf War who reported having symptoms of and 10 symptomatic non-veteran controls with lower back pain. 2. Participants and Methods: Subjects and controls were obtained from the Beth Israel Deaconess Medical Center and Harvard Medical School bio-repository. Control serum samples came from a separate study of non-veteran patients with chronic lower back pain who served as 'symptomatic' controls from one of the authors (EK). The proteins were separated using Western blot assay. 3. Results: Mean levels of autoantibodies in the subjects compared to controls were in descending order: CaMKII (9.27) > GFAP (6.60) > Tau (4.83) > Tubulin (4.41) > MAG (3.60) > MAP-2 (2.53) > MBP (2.50) > NFP (2.45) > S100B (1.03). 4. Conclusions: This study demonstrates a relationship between clinical condition, and the level of serum autoantibodies to nervous system-specific proteins. It is concluded that that these serum circulating autoantibodies may be used as biomarkers for confirming GWI when validated in larger samples. (Supported in part by DOD Contracts No. W81XWH-15-1-0641 and W81XWH-15-1-0640).